Cloning and characterization of human chemokine receptors

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Chemokines are a superfamily of proteins that have molecular masses of between 8 and 10 kDa and that display amino acid sequence identities of between 20 and 90%. They play a number of roles in inflammatory processes, including the selective recruitment and activation of leukocytes[1]. Their amino acid sequences contain four distinctive, conserved cysteine residues (Fig. 1). CXC (or α) chemokines, in which the first two cysteines are separated by one amino acid, are generally involved in neutrophil recruitment and activation and are implicated in acute inflammatory diseases. CC (or β) chemokines, in which the first two cysteines are adjacent, exert their effects on other leukocyte populations such as monocytes, T cells, eosinophils and basophils, and are implicated in chronic inflammatory conditions[2]. Lymphotactin[3] is a recently described protein that contains only two of the four conserved cysteine residues but otherwise retains overall sequence homology to other members of the chemokine family; this protein is probably the prototype of a third class of chemokines referred to as C chemokines.

The specific effects of chemokines on inflammatory cells are mediated by a family of G protein-coupled, seven transmembrane (7TM) receptors. Despite the fact that at least 21 human chemokines have been identified to date, only seven human chemokine receptors have been cloned. Two virally encoded chemokine receptors and the more distantly related erythrocyte Duffy antigen receptor (DARC), which also binds chemokines, have also been identified (Fig. 2). The ligand specificities and cellular distribution of these receptors are shown in Table 1.

CXC chemokine receptors

Two receptors for the CXC chemokine interleukin 8 (IL-8) have been identified. The IL-8 receptor A ($IL8_A$) was identified by expression cloning using I^{125}-labelled IL-8 (Ref. 4). The IL-8 receptor B ($IL8_B$) was identified in a dibutyryl cAMP-stimulated HL-60 cell DNA library by screening with a rabbit *N*-formylmethionyl-leucyl-phenylalanine (fMLP)-like receptor DNA probe[5]. Both $IL8_A$ and $IL8_B$ are predominantly expressed in polymorpho-

CXC chemokines

IL-8	SAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEIIVKLSD.GRELCLDPKENWVQRVVEKFLKRAENS
NAP-2	AELRCMCIKTTSG.IHPKNIQSLEVIGKGTHCNQVEVIATLKD.GRKICLDPDAPRIKKIVQKKLAGDESAD
ENA-78	AGPAAAVLRELRCVCLQTTQG.VHPKMISNLQVFAIGPQCSKVEVVASLKN.GKEICLDPEAPFLKKVIQKILDGGNKEN
GROα	ASVATELRCQCLQTLQG.IHPKNIQSVNVKSPGPHCAQTEVIATLKN.GRKACLNPASPIVKKIIEKMLNSDKSN
GROβ	APLATELRCQCLQTLQG.IHLKNIQSVKVKSPGPHCAQTEVIATLKN.GQKACLNPASPMVKKIIEKMLKNGKSN
GROγ	ASVVTELRCQCLQTLQG.IHLKNIQSVNVRSPGPHCAQTEVIATLKN.GKKACLNPASPMVQKIIEKILNKGSTN
IP-10	VPLSRTVRCTCISISNQPVNPRSLEKLEIIPASQFCPRVEIIATMKKKGEKRCLNPESKAIKNLLKAVSKEMSKRSP
GCP-2	GPVSAVLTELRCTCLRVTLR.VNPKTIGKLQVFPAGPQCSKVEVVASLKN.GKQVCLDPEAPFLKKVIQKILDSGNK
SDF-1	GKPVSLSYRCPCRFFESH.VARANVKHLKILN.TPNCALQIVARLKNNN.RQVCIDPKLKWIQEYLEKALNK
PF4	EAEEDGDLQCLCVKTTSQ.VRPRHITSLEVIKAGPHCPTAQLIATLKN.GRKICLDLQAPLYKKIIKKLLES
MIG	TPVVRKGRCSCISTNQGTIHLQSLKDLKQFAPSPSCEKIEIIATLKN.GVQTCLNPDSADVKELIKKWEKQVSQ

CC chemokines

RANTES	SPYSSDT.TPC.CFAYIARPLPRAHIKEYFYTSGK..CSNPAVVFVTRKN.RQVCANPEKKWVREYINSLEMS
I309	SKSMQVPFSRC.CFSFAEQEIPLRAILCYRNTSSI..CSNEGLIFKLKRG.KEACALDTVGWVQRHRKMLRHCPSKRK
MIP-1α	ASLAADTPTAC.CFSYTSRQIPQNFIADYFETSSQ..CSKPGVIFLTKRS.RQVCADPSEEWVQKYVSDLELSA
HCC1	TKTESSSRGPYHPSEC.CFTYTTYKIPRQRIMDYYETNSQ..CSKPGIVFITKRG.HSVCTNPSDKWVQDYIKDMKEN
MIP-1β	APMGSDPPTAC.CFSYTARKLPRNFVVDYYETSSL..CSQPAVVFQTKRS.KQVCADPSESWVQEYVYDLELN
MCP-1	QPDAINAPVTC.CYNFTNRKISVQRLASYRRITSSK.CPKEAVIFKTIVA.KEICADPKQKWVQDSMDHLDKQTQTPKT
Eotaxin	GPA..SVPTTC.CFNLANRKIPLQRLESYRITSGK..CPQKAVIFKTKLA.KDICADPKKKWVQDSMKYLDQKSPTPKP
MCP-2	QPDSVSIPITC.CFNVINRKIPIQRLESYTRITNIQ.CPKEAVIFKTKRG.KEVCADPKERWVRDSMKHLDQIFQNLKP
MCP-3	QPVGINTSTTC.CYRFINKKIPKQRLESYRRTTSSH.CPREAVIFKTKLD.KEICADPTQKWVQDFMKHLDKKTQTPKL

C chemokine

Lymphotactin	GVEVSDKRT.CVSLTTQRLPVSRIKTYTITEG...SLR.AVIFITKRGLK.VCADPQATWVRDVVRSMDRKSNTRNNMIQT

Fig. 1. Amino acid sequence alignment of human chemokines. Chemokines have been grouped as CXC, CC or C chemokines, with the conserved cysteine residues in red. ENA-78, epithelial-derived neutrophil attractant-78; IP-10, interferon γ inducible protein 10; GCP-2, granulocyte chemotactic protein 2; SDF-1, stomal cell derived factor 1; PF4, platelet factor 4; MIG, monokine induced by interferon γ.

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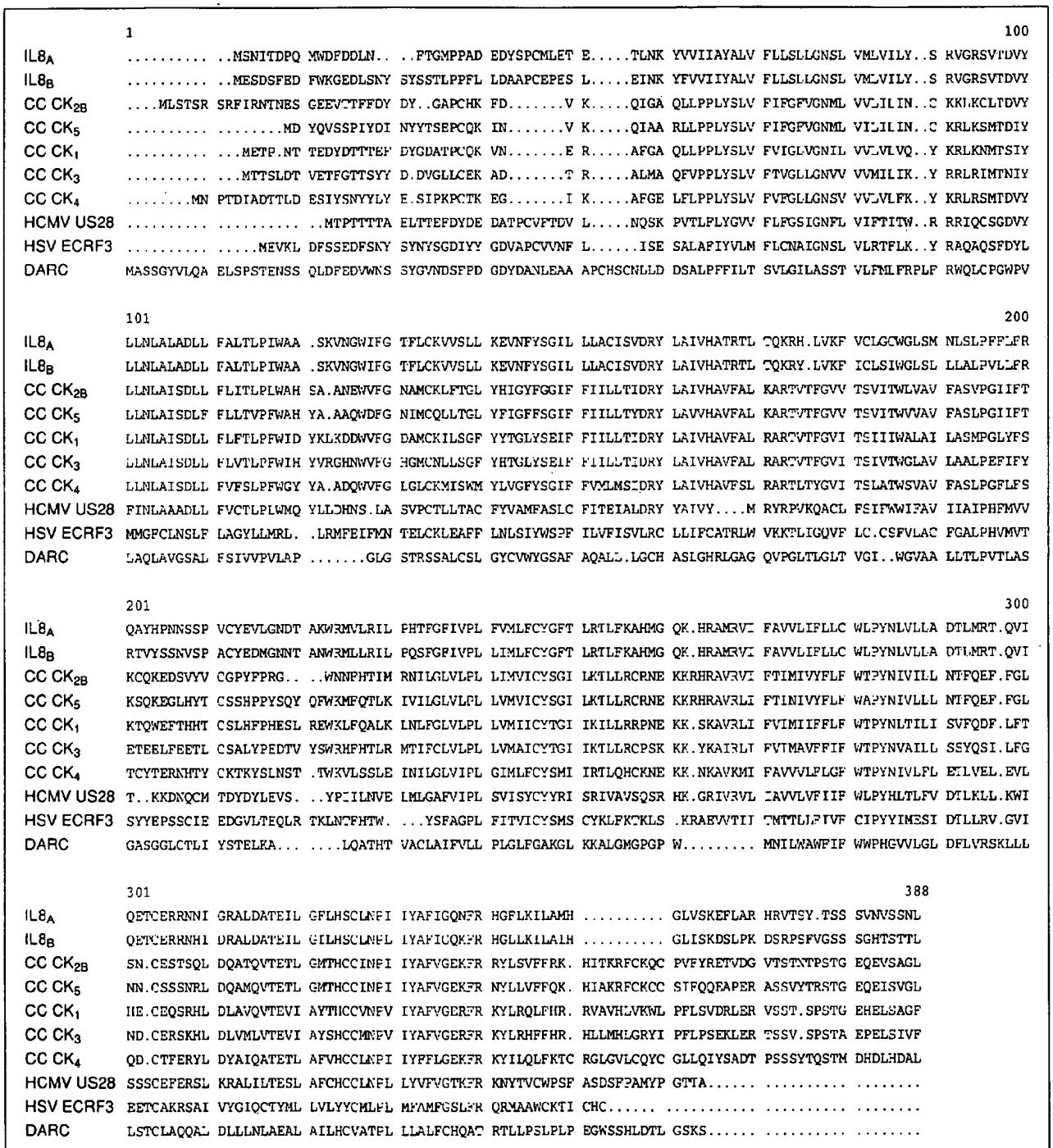

Fig. 2. Amino acid sequence alignment of chemokine receptors. Highly conserved residues are in red. Database accession codes for the sequences used in this alignment are M68932 for $IL8_A$, M73969 for $IL8_B$, L10918 for CC CK_1, U03882 for CC CK_{2B}, U28694 for CC CK_3, X85740 for CC CK_4, X91492 for CC CK_5, U01839 for DARC, X17403 for HCMV US28, S76368 for HSV ECRF3. To facilitate the alignment, CC CK_{2A} has not been included.

nuclear leukocytes and bind IL-8 at high affinity; however, the $IL8_A$ receptor is specific for IL-8, whereas $IL8_B$ can also bind other CXC chemokines at high affinity, such as neutrophil-activating peptide 2 (NAP-2) and growth related gene product α (GROα) or melanoma growth stimulating activity (MGSA) [and probably other chemokines containing a Glu-Leu-Arg (ELR in Fig. 1) sequence motif preceding the conserved CXC motif]. Neither of these receptors can bind CC chemokines.

CC chemokine receptors

Degenerate oligonucleotide PCR primers, based on the conserved

Table 1. The chemokine receptor family: summary of ligand-binding specificities and cellular distribution of human chemokine receptors

Receptor	Ligand (K_d)[a]	mRNA expression	Murine homologue	Refs
$IL8_A$	IL-8 (1.7 nM)	M, N, T	–	43
$IL8_B$	IL-8 (0.8 nM), GROα (1.2 nM), NAP-2	B, Bp, E, M, N, T,	mIL8	42,43
CC CK_1	MIP-1α (10 nM), RANTES (0.6 nM), MCP-3 (0.7 nM)	B, E, M, MΦ, N, T,	mMIP1α	6, 8, 9, 44
CC CK_{2B}	MCP-1 (0.26 nM), MCP-3 (6 nM)	B, Bp, M, T,	mJE-R	11, 12, 45
CC CK_3	Eotaxin	E, M,	mMIP1αRL2	44
CC CK_4	MIP-1α (14 nM), RANTES (9 nM), MCP-1	B, Bp, M, T,	mCC CK_{4A}	18
CC CK_5	MIP-1α, MIP-1β, RANTES		mMIP1α	45, 46
DARC	IL-8 (20 nM), GROα (24 nM), RANTES (42 nM), MCP-1 (34 nM)	EC (spleen, lung, brain and kidney)	mDARC	22
HCMV US28	RANTES (3.4 nM), MCP-1 (6.1 nM), MIP-1α (2.5 nM), MIP-1β (5.1 nM)	–	–	24
HSV ECRF3	GROα, NAP-2, IL-8	–	–	

[a]Nanomolar dissociation constants (K_d) are for recombinant receptors expressed in mammalian cell lines (where available); otherwise, ligand specificity is based on Ca^{2+} mobilization data obtained from *Xenopus laevis* oocytes.
B, B cell; Bp, basophil; E, eosinophil; EC, endothelial cell; M, monocyte; MΦ, macrophage; N, neutrophil; T, T cell.

sequences found in the IL8 receptors and other chemoattractant peptide receptors (such as those for C5a and fMLP), have been used in orphan receptor cloning strategies to identify CC chemokine receptors. Although such an approach has proved useful in identifying at least five distinct receptors (described below), one of the pitfalls of the method is that it cannot identify receptors that belong to a different class from 7TMs.

The CC CK_1 receptor was originally isolated from U937 or HL-60 cell lines[6,7] and was shown to be activated by macrophage inflammatory protein 1α (MIP-1α) and RANTES (regulated on activation normal T-cell expressed and secreted). Binding data reveal low nanomolar dissociation constants for MIP-1α (Ref. 6), RANTES (Ref. 8) and monocyte chemotactic protein 3 (MCP-3; Ref. 9). CC CK_2 was cloned from MonoMac6 cells and exists in two alternatively spliced forms, A and B, that differ in their cytoplasmic C-terminal domains[10]. Both forms of CC CK_2 mRNA are highly expressed in peripheral blood monocytes. HEK-293 cells stably expressing the receptor bind MCP-1 and MCP-3 at high affinity but surprisingly are unable to bind the closely related MCP-2 (Refs 11, 12). CC CK_3 has been cloned from activated peripheral blood mononuclear cells[13–15]. The high level of expression of CC CK_3 mRNA in eosinophils is consistent with the finding that it is a receptor for the eosinophil-specific chemoattractant eotaxin[15,16]. A fourth receptor, CC CK_4, has been identified in the human, immature, basophilic cell line KU-812 (Ref. 17). The receptor mRNA is highly expressed in T cells and IL-5 primed basophils. MIP-1α, RANTES and MCP-1 can activate this receptor when expressed in *Xenopus laevis* oocytes[18]. Direct binding of RANTES and MIP-1α has also been observed in HL-60 cells transiently expressing CC CK_4 (Ref. 18). More recently, a fifth CC chemokine receptor, CC CK_5 (or ChemR13), has been described[13,14,19]. CHO-K1 cells stably expressing CC CK_5 can respond to MIP-1α > MIP-1β and RANTES in a microphysiometer[19]. Although mRNA for this receptor has been detected in the promyeloblastic cell line KG-1A, no data have yet been published regarding its expression in normal cells.

Promiscu us receptors

DARC is a promiscuous chemokine receptor originally identified in erythrocytes[20] but also reported in restricted leukocyte populations and postcapillary, high-endothelial, venules[21]. It is unique as it binds a number of CXC chemokines (IL-8, MGSA and NAP-2) and CC chemokines (RANTES and MCP-1) at high affinity[22,23]. Despite the overlapping ligand-binding specificities with CXC and CC chemokine receptors, DARC shows less than 30% amino acid identity to these receptors. No signalling pathways have yet been described for the action of DARC.

Virally encoded receptors

Two virally encoded chemokine receptors have been reported. One, encoded by an open reading frame found in human cytomegalovirus US28 (and thus called HCMV US28), encodes a receptor that binds CC chemokines[24]. The other is a CXC chemokine receptor encoded by an open reading frame in Herpes saimiri virus ECRF3 (HSV ECRF3)[25]. While both receptors are capable of signal transduction, their significance *in vivo* is unclear. An antiviral role for chemokines in host defence is implied.

Chemokine-receptor-like orphan receptors

Degenerate, oligonucleotide-based, PCR cloning strategies have also

identified a large number of orphan receptors including G protein-coupled receptor 5 (Ref. 26), chemokine β receptor-like 1 (Ref. 27) [or V28 (Ref. 28)], leukocyte derived 7TM receptor (Ref. 29) and Burkitt lymphoma receptor 1 (Ref. 30), the mRNAs of which are generally highly expressed in leukocyte populations, notably T and B lymphocytes. Despite a high degree of sequence identity (30–50%) to known chemokine receptors, specific ligands for these receptors have yet to be identified. This may be because it is difficult to obtain a high level of expression of these receptors in mammalian cell lines, additional cofactors might be required, or it might be due simply to the fact that the physiological ligands have not yet been cloned. Possible roles of these proteins as viral receptors cannot be excluded yet.

Genomic localization

The genes encoding $IL8_A$ and $IL8_B$ co-localize on human chromosome 2q34–35 (Ref. 31), a region that also contains a pseudogene of $IL8_B$. The genes for CC chemokine receptors appear to be clustered on chromosome 3, with *ccckr1*, *2* and *5* found at 3p21 (Refs 7, 19) and *ccckr4* at 3p22 (Ref. 32). No chromosomal localization for the *ccckr3* gene has yet been reported. Interestingly, a number of genes encoding chemokine-receptor-like orphan receptors are also located in the region 3p21–22, including chemokine β receptor-like 1 and G protein-coupled receptor 5. The gene for DARC resides on chromosome 1q22–23 (Ref. 33).

Signalling pathways

Receptor activation by chemokines is generally sensitive to pertussis toxin, although a pathway insensitive to this toxin also exists for IL-8 that involves activation of Gα14 and Gα16 (Ref. 34). Activation of heterotrimeric G protein complexes results in dissociation of the α subunit from the βγ subunits and leads to activation of phospholipase C (PLC) β1 and β2. PLC activation results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce the second messengers inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG). These second messengers trigger a signalling cascade in which a variety of effectors are phoshorylated and activated, ultimately giving rise to diverse cellular responses such as chemotaxis, degranulation and respiratory burst. It appears that CC CK_2 is distinct in this respect since, although it couples to $G\alpha_i$, stimulation of the receptor with MCP-1 does not result in IP_3 production[11]. There is now evidence to suggest that multiple and distinct signalling pathways exist for chemokine receptors, depending on the cell type, receptor and ligand involved[35,36]. Recombinant chemokine receptors stably expressed in appropriate cell lines should prove to be useful tools for dissecting the operative pathways.

Chemokine receptors in disease

The presence of chemokines in a number of human disease pathologies with associated inflammation has been widely demonstrated (reviewed in Ref. 37). The use of specific anti-chemokine antibodies has been shown to curtail inflammation in a number of animal models (e.g. anti-MIP-1α in bleomycin-induced pulmonary fibrosis[38] and anti-IL-8 in reperfusion injury[39]). 'Knockout' mice for the gene encoding MIP-1α have no overt haematopoietic abnormalities but are resistant to myocarditis induced by Coxsackie virus and show reduced pneumonitis following infection with influenza virus, suggesting that MIP-1α is an important mediator of virus-induced inflammation[40].

Perhaps the clearest link of any chemokine receptor with disease is the relationship between DARC and malaria. DARC functions not only as a promiscuous chemokine receptor but also as a receptor for the malarial parasite *Plasmodium vivax*. DARC is absent on the erythrocytes of individuals in certain ethnic groups who are resistant to infection by *P. vivax*[20]. Yet it appears to be expressed normally elsewhere in these individuals. The repression of the gene expression in erythrocytes is due to a point mutation in the erythroid promoter[41].

The identification of murine homologues of chemokine receptors (based on sequence, tissue and cellular distribution, and functional similarities) will facilitate the construction of knockout mice that should then give insight into the biological relevance of chemokine receptors in disease. The murine $IL8_B$ homologue is the only receptor so far for which such published data exist: mice lacking this receptor show significantly reduced neutrophil migration to inflammatory sites compared with normal mice[42].

Closing remarks

Evidence is accumulating to indicate that chemokines and their receptors play a pivotal role in inflammation. Multiple chemokine receptors with considerable overlapping ligand specificities have now been identified and leukocytes generally express several different receptor types. The basis of this redundancy is unclear. *In vivo*, it is likely that both chemokine and specific chemokine receptor expression is regulated temporally and spatially. It also appears that different ligands may activate distinct signalling pathways at the same receptor. This suggests that specific receptors are likely to play a key role in a given disease state. Thus, the development of inhibitors targeted to distinct receptors will be important in the therapeutic intervention of inflammatory and viral diseases.

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Is there a 'lock' for all agonist 'keys' in 7TM receptors?

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It is generally assumed that the superfamily of rhodopsin-like seven transmembrane domain (7TM) receptors must have a common molecular-activation mechanism. This is based on the structural homology of the receptors, and the fact that they act through a common set of G proteins. The ligands for 7TM receptors cover all classes of chemical messengers: from metal ions and monoamines, purines and lipids to peptides and large proteins. Despite this great diversity in size and chemical composition, it has been assumed that these ligands still activate their respective receptors using a common mechanism. The initially characterized monoamine-binding site was the most obvious candidate for a general active site or a common 'lock' for all agonist 'keys'. Recent studies, for example on bradykinin and thrombin receptors, indicate that this may not be so, and evidence has begun to accumulate in favour of a receptor model with no requirement for a common active site.

The 'lock' for monoamine 'keys' is located deep within the main ligand-binding crevice

The binding site for catecholamines on adrenoceptors was characterized both by mutational mapping and by fluorescence spectroscopy in a pioneering series of papers[1,2]. The most crucial contact points are believed to be an Asp on TM-III (AspIII:08), two Ser residues on TM-V (SerV:09 and SerV:12), and a Phe on TM-VI (PheVI:17) – all located deep within the main ligand-binding crevice (see Figs 1 and 2). Most convincingly, the specific interaction between the amine function of the ligand and AspIII:08 on the receptor was shown by mutually complementary modifications on both the ligand and the receptor[3]. As presumed contact points for other monoamine ligands were subsequently identified in corresponding or neighbouring positions in their respective receptors, it was suggested that this deeply located pocket serves as a general interaction site, not only for monoamines, but for all agonists of the rhodopsin-like 7TM receptor family[4,5]. In the molecular models, ligands could reach down and touch this trigger area and thereby activate their respective receptors (e.g. for neuropeptides and glycoprotein hormones)[6,7]. Only through binding to this common lock would agonists be able to start a cascade of conformational alterations down through the TMs, which eventually would transfer the signal to the G protein[8].

However, results from mutational mapping experiments indicate that certain peptides such as substance P might in fact not contact the deeply located monoamine binding residues[9,10]. It was suggested that such peptides could, instead, activate their receptors merely by stabilizing an active conformation through ligand–

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